14. (New) The process of Claim 11, wherein the enterobacteria is *Enterobacter agglomerans* or *Klebsiella planticola*.

Claim 14 Repeated

REMARKS

Claims 1-2 and 6-14 are active in the present application. Support for Claims 6-14 is found in Claims 3-5 and the application as originally filed. No new matter is believed to have been added by these amendments. Favorable reconsideration is respectfully requested.

Applicants have now submitted a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. The sequence information recorded in the corresponding computer-readable Sequence Listing is identical to the paper copy of the substitute Sequence Listing. Support for all of the sequences listed in the substitute Sequence Listing is found in the present application as originally filed. No new matter is believed to have been introduced by the submission of the substitute Sequence Listing and the corresponding computer-readable Sequence Listing.

The objections to Claims 4 and 5 is obviated by the cancellation of these claims.

The rejection of Claims 3-4 under 35 U.S.C. §112 is obviated by the cancellation of the claims.

The rejections of Claims 1-4 under 35 U.S.C. §112, first paragraph are respectfully traversed.

The Examiner alleges that the specification only enables and describes an enterobacteria containing a DNA encoding *Brevibacterium lactofermentum* citrate synthase, but does not provide enablement for an enterobacteria containing DNA encoding any coryneform bacterial citrate synthase. Applicants respectfully disagree and direct the Examiner's attention to the relevant section of the MPEP which discusses enablement:

> The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation MPEP § 2164.01

Applicants submit that one of skill in the art could obtain genes encoding citrate synthase other than that of *Brevibacterium lactofermentum* at the time the application was filed. Coupled with the standard knowledge available in the art the present specification disclosure clearly enables the skilled artisan to make and/or use the invention.

As illustrative of the knowledge available in the art, Applicants submit herewith a copy of *Microbiology* (1994), 140, 1817-1828 (referenced on page 18, line 17 in the present specification), which describes the nucleotide sequence of *Corynebacterium glutamicum* gltA gene encoding citrate synthase. This manuscript further describes amino acid sequences of citrate synthases from *E. coli*, *P. aeruginosa*, *R. prowazakii* and *Bacillus sp.* C4, and aligns these amino acid sequences revealing approximately seven highly conserved regions (see page 1824, right column, last paragraph). Therefore, coryneform bacterial citrate synthase genes other than the *Brevibacterium lactofermentum* gene can be cloned by conventional methods such as hybridization or PCR using fragments or oligonucleotides corresponding to the above conserved regions from bacterial chromosomal DNA.

Applicants respectfully direct the Examiner's attention to MPEP § 2164.01(b): "As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. § 112 is satisfied. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). In the present application, the Applicants have described on page 18, line 11 the isolation of the gltA gene by PCR using specific nucleotide primers

shown in SEQ ID NOS:1 and 2 and methods of introducing the citrate synthase genes into bacteria (see pages 18-20). Thus, the instant claims are clearly enabled.

With respect to the written description rejection, Applicants direct the Examiner's attention to MPEP § 2163.02:

> An objective standard for determining compliance with the written description requirement is, "does the description clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed." *In re Gostelli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989).

As discussed at length above, the present application provides a clear description of the source of the citrate synthase gene, methods of isolating the gene and methods of introducing the gene into a bacteria. Furthermore, the skilled artisan would understand the conserved regions of citrate synthase as disclosed in the *Microbiology* (1994) manuscript referenced on page 18, line 17 and utilize this information with the disclosure in the present specification. Thus, the specification provides the skilled artisan a clear indication that the inventors had possession of the claimed invention at the time the application was filed.

Accordingly, withdrawal of both grounds of rejection under 35 U.S.C. § 112, 1st paragraph is respectfully requested.

The rejection of Claims 1-4 under 35 U.S.C. §103(a) over Katsumata et al in view of Moriya et al is respectfully traversed.

The present application claims priority to Japanese Patent Application No. 10-297350 filed October 19, 1998. Applicants have previously submitted a request for priority accompanied with the certified priority document on October 19, 199. In order to perfect priority to the October 19, 1998 priority date Applicants submit herewith a certified English translation of the JP 10-297350. The effective date of Moriya et al is March 18, 1999 which

is after the priority date of the instant application. Accordingly, withdrawal of this ground of rejection is respectfully requested.

Applicants submit that the present application is now in condition for allowance. Early notification of such allowance is earnestly solicited.

Respectfully submitted,

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Marked-Up Copy
Serial No: 09/419, 611
Amendment Filed on:
_____JULY 13, 2001___

IN THE SPECIFICATION

Substitute Sequence Listing (New).

IN THE CLAIMS

Claims 3-5 (Cancelled).

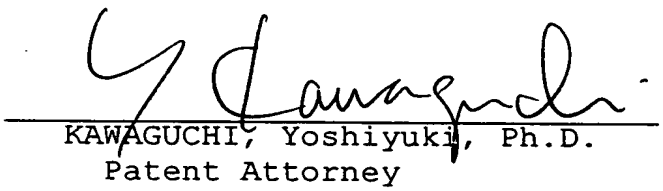
Claims 6-14 (New).

VERIFICATION OF TRANSLATION

Japanese Patent Application No.Hei 10- 297350
filed on October 19, 1998

I, Yoshiyuki KAWAGUCHI, a citizen of Japan whose address is c/o SERA, TOYAMA, MATSUKURA, KAWAGUCHI & KOIWAI, Yokoyama Bldg., 6th Floor, 4-10, Higashi Nihonbashi 3-chome, Chuo-ku, Tokyo, 103-0004 Japan, am a translator of the document attached and I state that the following is a true translation to the best of my knowledge and belief.

Signature of translator:



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Patent Attorney
SERA, TOYAMA, MATSUKURA,
KAWAGUCHI & KOIWAI

Signed at Tokyo, Japan,
This 27th day of June, 2001

PATENT OFFICE
JAPANESE GOVERNMENT

This is to certify that annexed is a true copy of the following application as filed with this office.

Date of Application: October 19, 1998

Application No.: Patent Application No. Hei 10-297350
(297350/1998)

Applicant(s): Ajinomoto Co., Ltd.

June 11, 1999

Commissioner,
Patent Office

Takeshi Isayama (Seal)

Cert. No. 10-3037783

[Document Name] Application for patent

[Number] P-5935

[Application filed] October 19, 1998

[To] Commissioner of Patent Office

[International Class] C12P 13/06

[Title of the Invention] L-Glutamic Acid Producing Bacterium and Process for Producing L-Glutamic Acid

[Number of Claims] 5

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[Indication of Official Fees]

[ledger number]	012092	
[amount paid]	21,000	

[List of attached documents]

[document name]	Specification	1
[document name]	Drawing	1
[document name]	Abstract	1

[Requirement of proof] Yes

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[document name]	Specification	1
[document name]	Drawing	1
[document name]	Abstract	1

[Requirement of proof] Yes

[Name of Document] Specification

[Title of the Invention] L-GLUTAMIC ACID PRODUCING BACTERIUM AND PROCESS FOR PRODUCING L-GLUTAMIC ACID

[Claims]

1. A microorganism belonging to enterobacteria and having L-glutamic acid productivity, into which a citrate synthase gene derived from a coryneform bacterium is introduced.

2. The microorganism of claim 1 wherein the coryneform bacterium is *Brevibacterium lactofermentum*.

3. The microorganism of any one of claims 1 or 2 wherein the microorganism belonging to enterobacteria which a bacterium belonging to the genus *Enterobacter* or *Klebsiella*.

4. The microorganism of claim 3 wherein the bacterium belongs to *Enterobacter agromellans* or *Klebsiella planticola*.

5. A process for producing L-glutamic acid comprising the steps of culturing the microorganism of any one of the cliams 1 to 4 in a liquid medium to produce and accumulate L-glutamic acid in the medium and collecting the L-glutamic acid from the medium.

[Description of Invention]

[Field of Industrial Application]

The present invention relates to a new L-glutamic

acid producing bacterium and a process for producing L-glutamic acid by a fermentation method using the same. L-glutamic acid is an important amino acid as a food, medicament or the like.

[Prior Art]

Heretofore, L-glutamic acid has been produced by a fermentation method using mainly so-called a coryneform L-glutamic acid producing bacterium belonging to the genus *Brevibacterium*, *Corynebacterium* or *Microbacterium* or a variant thereof (Amino Acid Fermentation, Gakkai Shuppan Center, pp. 195-215, 1986). Known processes for producing L-glutamic acid by a fermentation method using other strains include one using a microorganism belonging to the of *Bacillus*, *Streptomyces* or *Penicillium* (U.S. Patent No. 3,220,929), one using a microorganism belonging to the genus *Pseudomonas*, *Arthrobacter*, *Serratia* or *Candida* (U.S. Patent No. 3,563,857), one using a microorganism such as a bacterium belonging to the genus *Bacillus*, *Pseudomonas*, *Serratia* or *Aerobacter aerogenes* (currently *Enterobacter aerogenes*) (Examined Japanese Patent Publication No. 32-9393), one using a mutant of *Escherichia coli* (Japanese Laid-open Patent Application No. 5-244970) and the like.

The productivity of L-glutamic acid has been considerably improved by the breeding of the above

microorganisms or the improvement of the production processes. To meet growing demand for L-glutamic acid, the development of a more inexpensive and efficient process for producing L-glutamic acid is desired.

In view of the above situation, the inventors of the present invention has investigated and studied microorganisms having L-glutamic acid productivity broadly. As a result, it has been found that a microorganism having high L-glutamic acid productivity can be obtained by increasing the activity of an enzyme which catalyzes the L-glutamic acid biosynthesis reaction (citrate synthase, phosphoenolpyruvate carboxylase, glutamate dehydrogenase) of a microorganism belonging to the genus *Enterobacter*, *Serratia*, *Klebsiella* or *Erwinia* (Japanese Laid-open Patent Application Nos. 10-224909 and 10-297129).

The inventors have also found that a microorganism having high L-glutamic acid productivity is obtained by enhancing the activity of each of these enzymes by introducing a gene coding citrate synthase (hereinafter sometimes abbreviated as "CS") and phosphoenolpyruvate carboxylase derived from the genus *Escherichia* into a valine sensitive strain belonging to the genus *Escherichia* (WO 97/08294).

Meanwhile, it has been reported that the

introduction of a gene (CS gene) coding citrate synthase derived from *Escherichia coli* or *Corynebacterium glutamicum* is effective in improving the L-glutamic acid productivity of *Corynebacterium* or *Brevibacterium* (Examined Japanese Patent Publication No. 7-121228). When these coryneform bacteria were used as a host, the introduction of a CS gene derived from *Corynebacterium glutamicum* of the same species as the host showed a slightly higher effect than the introduction of a CS gene derived from *Escherichia coli* but there was not seen a marked difference between them.

As described above, it has been known that a CS gene is introduced into various microorganisms to improve L-glutamic acid productivity. However, there has not been known an example where a CS gene derived from a coryneform bacterium is introduced into a microorganism belonging to enterobacteria such as a bacterium belonging to the genus *Escherichia*.

[Problem to be solved by the invention]

It is an object of the present invention to find a new L-glutamic acid producing bacterium having L-glutamic acid productivity in order to develop an inexpensive and efficient process for producing L-glutamic acid.

[Means to Solve the Problem]

The inventors of the present invention have bred enterobacteria by introducing a gene to improve their productivity of L-glutamic acid. Generally, it has been considered that a better effect is obtained by using a endogenous gene of a host or a gene derived from a microorganism which is a relative of the host than by introducing a heterogeneous gene when the host has a target gene for the breeding of a microorganism by gene amplification. However, the inventors of the present invention have found it much more effective, for enterobacteria, in improving the L-glutamic acid productivity of a microorganism to introduce a CS gene derived from a coryneform bacterium than to introduce a CS gene derived from a microorganism of the same species as the enterobacteria. The present invention has been accomplished based on this finding.

That is, the present invention provides:

(1) a microorganism belonging to enterobacteria and having L-glutamic acid productivity, into which a citrate synthase gene derived from a coryneform bacterium is introduced,

(2) the microorganism of above (1) wherein

the coryneform bacterium is *Brevibacterium lactofermentum*,

(3) the microorganism of (1) or (2) wherein the microorganism belonging to enterobacteria which a bacterium belonging to the genus *Enterobacter* or *Klebsiella*,

(4) the microorganism of (3) wherein the bacterium belongs to *Enterobacter agromellans* or *Klebsiella planticola*, and

(5) a process for producing L-glutamic acid comprising the steps of culturing the microorganism of any one of the above (1) to (4) in a liquid medium to produce and accumulate L-glutamic acid in the medium and collecting the L-glutamic acid from the medium.

[Embodiment of the Invention]

The present invention will be described in detail below.

<1> microorganism of the present invention

The microorganism belonging to enterobacteria of the present invention is not particularly limited if it belongs to enterobacteria which can be conferred or improved L-glutamic acid productivity by introducing a CS gene derived from a coryneform bacterium. The

microorganism is exemplified by a bacterium belonging to the genus *Enterobacter*, *Klebsiella*, *Serratia*, *Erwinia* or *Escherichia*. Out of these, bacteria belonging to the genus *Enterobacter* or *Klebsiella* are preferred. Illustrative examples of the bacteria are described below but the microorganism of the present invention is not limited to these examples.

Examples of the microorganism belonging to the genus *Enterobacter* that can be used for the present invention are listed below.

Enterobacter agglomerans

Enterobacter aerogenes

Enterobacter amnigenus

Enterobacter asburiae

Enterobacter cloacae

Enterobacter dissolvens

Enterobacter gergoviae

Enterobacter hormaechei

Enterobacter intermedius

Enterobacter nimipressuralis

Enterobacter sakazakii

Enterobacter taylorae

More preferably, those bacterial strains listed bel ow can be mentioned:

Enterobacter agglomerans AJ13355

Serratia liquefacience ATCC 14460

The *Enterobacter agglomerans* AJ13355 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on February 19, 1998, and received an accession number of FERM P-16644. The *Enterobacter agglomerans* ATCC 12287, and the *Serratia liquefacience* ATCC 14460 can be distributed from ATCC.

The *Enterobacter agglomerans*. AJ13355 strain is a strain isolated from soil in Iwata-shi, Shizuoka, Japan.

Physiological properties of AJ13355 are as follows.

(1) Gram stain: Negative

(2) Aerophobicity: Facultative anaerobe

(3) Catalase: Negative

(4) Oxidase: Positive

(5) Nitrate reduction ability: Negative

(6) Voges-Proskauer reaction: Positive

(7) Methyl Red test: Negative

(8) Urease: Negative

(9) Indole production: Positive

(10) Motility: Present

(11) Hydrogen sulfide production in TSI culture medium: Slightly active

(12) β-Galactosidase: Positive

(13) Sugar assimilability:

Arabinose: Positive

Sucrose: Positive

Lactose: Positive

Xylose: Positive

Sorbitol: Positive

Inositol: Positive

Trehalose: Positive

Maltose: Positive

Melibiose: Positive

Adonitol: Negative

Raffinose: Positive

Salicin: Negative

Melibiose: Positive

(14) Glycerose assimilability: Positive

(15) Organic acid assimilability:

Citric acid: Positive

Tartaric acid: Negative

Gluconic acid: Positive

Acetic acid: Positive

Malonic acid: Negative

(16) Arginine dehydratase: Negative

(17) Ornithine decarboxylase: Negative

(18) Lysine decarboxylase: Negative

(19) Phenylalanine deaminase: Negative

(20) Chromogenesis: Yellow

(21) Gelatin liquefaction ability: Positive

(22) Growth pH: Not good growth at pH 4, good growth at pH 4.5-7

(23) Growth temperature: Good growth at 25°C, good

growth at 30°C, good growth at 37°C, growth is possible at 42°C, no growth at 45°C

From these bacteriological properties, AJ13355 is determined to be *Enterobacter agglomerans*.

Examples of the microorganism belonging to the genus *Klebsiella* that can be used for the present invention are listed below.

Klebsiella planticola

Klebsiella terrigena

More preferably, the examples of the microorganism include *Klebsiella planticola* AJ13399.

The *Klebsiella planticola* AJ13399 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on February 19, 1998, and received an accession number of FERM P-16646.

The *Klebsiella planticola* AJ13399 strain is a strain isolated from soil in Sapporo-shi, Hokkaido, Japan.

Physiological properties of AJ13399 are as follows.

(1) Cell morphology: Rod-shaped

(2) Motility: Absent

(3) Spore formation: Absent

(4) Colony morphology on LabM nutrient agar: Circular, smooth surface, cream in color, even, raised, and glistening

(5) Glucose OF test: Positive for fermentability

(6) Gram stain: Negative

(7) Aerophobicity: Facultative anaerobe

(8) Catalase: Positive

(9) Oxidase: Negative

(10) Urease: Positive

(11) Cytochrome oxidase: Negative

(12) β-Galactosidase: Positive

(13) Arginine dehydratase: Negative

(14) Ornithine decarboxylase: Negative

(15) Lysine decarboxylase: Positive

(16) Tryptophan deaminase: Negative

(17) Voges-Proskauer reaction: Positive

(18) Indole production: Positive

(19) Hydrogen sulfide production in TSI culture medium: Negative

(20) Citric acid assimilability: Positive

(21) m- Hydroxybenzene acid assimilability: Negative

(22) Gelatin liquefaction ability: Negative

(23) Production of acid from sugar

Glucose: Positive

Mannitol: Positive

Rhamnose: Positive

Arabinose: Positive

Sucrose: Positive

Sorbitol: Positive

Inositol: Positive

Melibiose: Positive

Amygdalin: Positive

Adonitol-peptone-water: Positive

Cellobiose-peptone-water: Positive

Dulcitol-peptone-water: Negative

Raffinose-peptone-water: Positive

(24) Growth temperature: Good growth at 37°C, no growth at 45°C

From these bacteriological properties, AJ13399 is determined to be *Klebsiella planticola*.

Examples of the microorganism belonging to the genus *Serratia* that can be used for the present invention are listed below.

Serratia liquefacience

Serratia entomophila

Serratia ficaria

Serratia fonticola

Serratia grimesii

Serratia proteamaculans

Serratia odorifera

Serratia plymuthica

Serratia rubidaea

More preferably, *Serratia liquefacience* ATCC 14460 may be exemplified. *Serratia liquefacience* ATCC 14460 can be distributed from ATCC.

Examples of the microorganism belonging to the genus *Erwinia* that can be used for the present invention

are listed below.

Erwinia herbicola

Erwinia ananas

Erwinia cacticida

Erwinia chrysanthemi

Erwinia mallotivora

Erwinia persicinus

Erwinia psidii

Erwinia quercina

Erwinia rhapontici

Erwinia rubrifaciens

Erwinia salicis

Erwinia uredovora

More preferably, *Erwinia herbicola* IAM1595 is exemplified. *Erwinia herbicola* IAM1595 can be distributed from the Institute of Molecular and Cellular Biosciences, the University of Tokyo.

Examples of the microorganism belonging to the genus *Escherichia* that can be used for the present invention include *Escherichia coli*.

More preferably, *Escherichia coli* having valine resitance, for example, the following strains may be exemplified.

Eschericha coli K-12 (ATCC10798)

Escherichia coli B (ATCC11303)

Escherichia coli W (ATCC9637)

Eschericha coli K-12 (ATCC10798), *Escherichia coli*

B (ATCC11303) and *Escherichia coli* W (ATCC9637) can be distributed from ATCC.

It should be noted that, the sugar metabolism by bacteria belonging to the genera *Enterobacter*, *Klebsiella*, *Serratia*, *Erwinia* and *Escherichia* such as those mentioned above is achieved via the Embden-Meyerhof pathway, and pyruvic acid produced in that pathway is oxidized in the tricarboxylic acid cycle as for aerobic condition. L-glutamic acid is biosynthesized from α-ketoglutaric acid which is an intermediate of the tricarboxylic acid cycle by GDH or glutamine synthetase/glutamate synthase. Thus, these microorganisms share the same biosynthetic pathway for L-glutamic acid, and microorganisms mentioned above are encompassed within a single conception according to the present invention. Therefore, microorganisms belonging to the enteric bacteria other than species and strains mentioned above also fall within the scope of the present invention.

The microorganism of the present invention is a microorganism belonging to the enteric bacteria and having L-glutamic acid productivity. The term "having L-glutamic acid production ability" as herein used means to have ability for accumulating L-glutamic acid in culture medium during cultivation. This L-glutamic acid production ability may be either one possessed by a wild-type strain as its property, or one imparted or

enhanced by breeding. Microorganism, which can be imparted L-glutamic acid productivity by being introduced *gltA* gene, may be also used. The microorganism belonging to the enteric bacteria and having the L-glutamic acid production ability include, for example, such microorganisms having increased activity of one or more enzymes catalyzing one or more reactions for the biosynthesis of L-glutamic acid, and such microorganisms having decreased activity of an enzyme catalyzing a reaction branching from the pathway for L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid, or lacking the activity. The microorganism further includes those having increased activity of one or more enzymes catalyzing one or more reactions for the biosynthesis of L-glutamic acid, and decreased activity of an enzyme catalyzing a reaction branching from the pathway for L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid, or lacking the activity.

The "coryneform bacteria" which can be a souce for *gltA* gene being introduced into the enteric bactria includes bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus Corynebacterium at present (*Int. J. Syst. Bacteriol., 41*, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform L-glutamic

acid-producing bacteria include the followings.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium (*Corynebacterium glutamicum*)

Corynebacterium melassecola

Corynebacterium thermoaminogenes

Corynebacterium herculis

Brevibacterium divaricatum (*Corynebacterium glutamicum*)

Brevibacterium flavum (*Corynebacterium glutamicum*)

Brevibacterium immariophilum

Brevibacterium lactofermentum (*Corynebacterium glutamicum*)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Brevibacterium album

Brevibacterium cerinum

Microbacterium ammoniaphilum

Specifically, the following strains of these bacteria are exemplified:

Corynebacterium acetoacidophilum ATCC13870

Corynebacterium acetoglutamicum ATCC15806

Corynebacterium alkanolyticum ATCC21511

Corynebacterium callunae ATCC15991

Corynebacterium glutamicum ATCC13020, 13032, 13060

Corynebacterium lilium (*Corynebacterium glutamicum*) ATCC15990

Corynebacterium melassecola ATCC17965

Corynebacterium thermoaminogenes AJ12340(FERM BP-1539)

Corynebacterium herculis ATCC13868

Brevibacterium divaricatum (*Corynebacterium glutamicum*) ATCC14020

Brevibacterium flavum (*Corynebacterium glutamicum*) ATCC13826, ATCC14067

Brevibacterium immariophilum ATCC14068

Brevibacterium lactofermentum (*Corynebacterium glutamicum*) ATCC13665, ATCC13869

Brevibacterium roseum ATCC13825

Brevibacterium saccharolyticum ATCC14066

Brevibacterium thiogenitalis ATCC19240

Brevibacterium album ATCC15111

Brevibacterium cerinum ATCC15112

Microbacterium ammoniaphilum ATCC15354

A *gltA* gene derived from a coryneform bacterium can be obtained by isolating a DNA fragment which complement auxotrophy of a bacterium laking CS activity such as a mutant of a coryneform bacterium from the chromosome DNA of the coryneform bacterium. The

nucleotide sequence of the *gltA* gene of the coryneform bacterium is made known (*Microbiology, 140,* 1817-1828 (1994)). Therefore the *gltA* gene can be obtained by PCR method using the chromosome DNA as a template and primers which are synthesized based on the nucleotide sequence. The primers are exemplified by oligonucleotides having nucleotide sequence shown in SEQ ID NOS: 1 and No. 2.

To introduce a CS gene derived from a coryneform bacterium into the microorganism belonging to enterobacteria, the CS gene may be cloned on an appropriate plasmid and the above starting parent strain which serves as a host may be transformed with the obtained plasmid. The number of copies of the CS gene (hereinafter abbreviated as "*gltA* gene") in the cell of the transformant is increased with the result of enhancement of CS activity.

While the plasmid is not particularly limited so long as it can autonomously replicate in a microorganism belonging to the enteric bacteria, examples of the plasmid include, for example, pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219, pMW218 and the like. Other than these plasmids, phage DNA vectors can also be used.

The introduction of the *gltA* gene can be realized

by making the *gltA* gene present on the chromosome DNA of the above starting parent strain which serves as a host, preferably in multiple copies. To introduce the *gltA* gene into the chromosome DNA of the microorganism belonging to enterobacteria in multiple copies, a sequence present in the chromosome DNA in multiple copies such as repetitive DNA or inverted repeat present at a terminal region of a transposable element can be used. Alternatively, the *gltA* gene may be introduced into the chromosome DNA in multiple copies by inserting the *gltA* gene in a transposon and transposing the transposon. The number of copies of the *gltA* gene in the cell of the transformant increases, thereby enhancing CS activity.

Transformation may be performed in accordance with, for example, a method of D. A. Morrison (*Methods in Enzymology, 68*, 326 (1979)) or a method in which recipient cells are treated with calcium chloride to increase permeability for DNA (Mandel, M. and Higa, A., *J. Mol. Biol., 53*, 159 (1970)).

The *gltA* gene which is to be introduced may have a promoter suitable for the cell of a microorganism belonging to enterobacteria, such as *lac*, *trp*, or P_L in place of an inherent promotor of the *gltA* gene.

Techniques such as cloning of a gene, digestion

and ligation of DNA and transformation method are described in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

In the microorganism of the present invention, the activity of an enzyme which catalyzes the biosynthesis of L-glutamic acid other than CS may be enhancing in addition to the introduction of a *gltA* gene derived from a coryneform bacterium. Illustrative examples of the enzyme for catalyzing the biosynthesis of L-glutamic acid include glutamate dehydrogenase (GDH), glutamine synthase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, phosphoenolpyruvate carboxylase (PEPC), pyruvate dehydrogenase, pyruvate kinase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, fructose bisphosphate aldolase, phosphofructokinase, glucose phosphate isomerase and the like.

The activity of an enzyme which catalyzes a reaction for generating a compound other than L-glutamic acid by branching off from the biosynthetic pathway of L-glutamic acid may be decreased or lost. Illustrative examples of the enzyme which catalyzes a reaction for generating a compound other than L-glutamic acid by

branching off from the biosynthetic pathway of L-glutamic acid include α-ketoglutarate dehydrogenase (αKGDH), isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroximate synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline dehydrogenase and the like. Out of these enzymes, αKGDH is preferred.

The genes coding for PEPC and GDH can each be obtained from a chromosome DNA of the aforementioned microorganisms by isolating a DNA fragment complementing auxotrophy of a variant strain lacking the PEPC or GDH activity. Alternatively, because the nucleotide sequences of these genes of bacteria of the genus *Escherichia* or *Corynebacterium* have already been elucidated (*Biochemistry, 22,* 5243-5249 (1983); *J. Biochem. 95,* 909-916 (1984); *Gene, 27,* 193-199 (1984); *Mol. Gen. Genet. 218,* 330-339 (1989) and *Molecular Microbiology, 6,* 317-326 (1992)), the genes can be obtained by PCR using a primer synthesized based on each of the elucidated nucleotide sequences, and the chromosome DNA as a template.

In order to obtain such decrease or absence of enzyme activity as mentioned above in a microorganism belonging to the enteric bacteria, a mutation causing the decrease or absence of the enzyme activity can be

introduced into a gene encoding the enzyme by a conventional mutagenesis technique or genetic engineering technique

Examples of the mutagenesis technique include, for example, the method utilizing irradiation of X-ray or ultraviolet light, the method utilizing treatment with a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine and the like. The site of gene to which a mutation is introduced may be a coding region encoding an enzyme protein, or an expression control region such as a promoter.

Examples of the genetic engineering technique include, for example, genetic recombination, genetic transduction, cell fusion and the like. For example, a drug resistance gene is inserted into a target gene to produce a functionally inactivated gene (disrupted gene). Then, this deletion type gene is introduced into a cell of a microorganism belonging to the enteric bacteria, and the target gene on a chromosome is replaced with the deletion type gene by homologous recombination (gene disruption).

Whether a microorganism has decreased activity of a target enzyme or lacks the activity, or degree of the decrease of the activity can be determined by measuring the enzyme activity of bacterial cell extract or purified fraction of a candidate strain, and comparing it with that of a wild-type strain. For example, the

αKGDH enzymatic activity can be measured by the method of Reed et al. (L.J. Reed and B.B. Mukherjee, Methods in Enzymology 1969, 13, p.55-61).

For some enzymes, a target mutant may be selected by phenotype of the mutant. For example, a mutant whose αKGDH activity is lost or decleased cannot grow or has a large decrease in its growth rate in a minimal medium containing glucose or a minimal medium containing acetic acid or L-glutamic acid as the only carbon source. However, normal growth is made possible by adding succinic acid or L-lysine, L-methionine and diaminopimelic acid to a minimal medium containing glucose under the same conditions. It is possible to carry out a screening for a mutant whose αKGDH activity is lost or decreased using this phenomenon as an index.

A method for producing a *Brevibacterium lactofermentum* strain lacking the αKGDH gene based on homogenous recombination is detailed in WO95/34672, and a similar method can be used for microorganisms belonging to the enteric bacteria.

Examples of the mutant strain that lack the αKGDH activity or have decreased activity thereof obtained as described above are *Enterobacter agglomerans* AJ13356 and *Klebsiella planticola* AJ13410. The strain *Enterobacter agglomerans* AJ13356 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of

International Trade and Industry on February 19, 1998 as accession number of FERM P-16645. The strain *Klebsiella planticola* AJ13410 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on February 19, 1998 as accession number of FERM P-16647

L-Glutamic acid can be produced and accumulated in a liquid culture medium by culturing the microorganism belonging to the enteric bacteria and being introduced *gltA* gene derived from coryneform bacteria in the medium.

The medium may be an ordinary nutrient medium containing a carbon source, nitrogen source, and inorganic salts, as well as organic nutrients such as amino acids, vitamins and the like, as required. It can be a synthetic medium or a natural medium. Any carbon sources and nitrogen sources can be used for the culture medium so long as they can be utilized by the microorganism to be cultured.

The carbon source may be a saccharide such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysates, molasses and the like. Further, an organic acid such as acetic acid and citric acid may also be used alone or in combination with other carbon sources.

The nitrogen source may be ammonia, ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium

chloride, ammonium phosphate, and ammonium acetate, nitrates and the like.

As organic trace nutrients, amino acids, vitamins, fatty acids, nucleic acids, materials containing them such as peptone, casamino acid, yeast extract, and soybean protein decomposition products and the like are used, and when an auxotrophic variant which requires an amino acid or the like for its growth is used, it is necessary to complement the nutrient required.

As the inorganic salt, phosphates, magnesium salts, calcium salts, iron salts, manganese salts and the like are used.

As for the culture conditions, cultivation is performed under aerobic condition at a temperature of 20-42°C and a pH of 4-8. The cultivation can be continued for 10 hours to 4 days to accumulate a considerable amount of L-glutamic acid in the liquid culture medium.

After the completion of the cultivation, L-glutamic acid accumulated in the culture medium may be collected by a known method. For example, it can be isolated by a method comprising concentrating the medium after removing the cells to crystallize the product, ion exchange chromatography or the like.

According to the present invention, since a microorganism belonging to enterobacteria can be efficiently conferred L-glutamic acid productivity, it

is assumed that higher productivity can be conferred on the microorganisms by conventionally known breeding techniques for coryneform L-glutamic acid producing bacteria. Studies on culture conditions and the like are expected to lead to the development of an inexpensive and efficient process for producing L-glutamic acid.

[Examples]

The present invention will be explained more specifically with reference to the following examples.

(1) Construction of plasmid having *gltA* gene

A plasmid having the *gltA* gene derived from *Brevibacterium lactofermentum* was constructed as follows. PCR was performed by using primers having the nucleotide sequences represented in SEQ ID NOS: 1 and 2 selected based on the nucleotide sequence of the *gltA* gene of *Corynebacterium glutamicum* (Microbiology, 1994, 140, 1817-1828), and a chromosome DNA of *Brevibacterium lactofermentum* ATCC 13869 as a template to afford a *gltA* gene fragment of about 3 kb. This fragment was inserted into the plasmid pHSG399 (purchased from Takara Shuzo) digested with SmaI to afford a plasmid pHSGCB (Figure 1). Then, the pHSGCB was digested with HindIII, and an excised *gltA* gene fragment of about 3 kb was inserted into the plasmid pMW218 (purchased from Nippon Gene) digested with HindIII to afford a plasmid pMWCB (Figure

1). Expression of the *gltA* gene by the resulting plasmid pMWCB was confirmed by determination of enzyme activity in the *Enterobacter agglomerans*. AJ13355 strain.

(2) Construction of plasmid having *gltA* gene derived from *E. coli*

As a control, a placmid having *gltA* gene derived from *Escherichia coli* was constructed as follows. The plasmid pTWVC having the *gltA* gene derived from *Escherichia coli* (WO97/08294) was digested with HindIII and EcoRI, and the resulting DNA fragment having the *gltA* gene was purified and collected, and introduced into the HindIII-EcoRI site of the plasmid pMW219 to afford a plasmid pMWC (Figure 2). Expression of the *gltA* gene by the resulting plasmid pMWC was confirmed by determination of enzyme activity and complementation of auxotrophic strain of *E. coli* laking *gltA* gene.

(3) Introduction of *gltA* gene into *Enterobacter agglomerans* and *Klebsiella planticola* and production of L-glutamic acid

The strains *Enterobacter agglomerans* AJ13355 and the *Klebsiella planticola* AJ13399 were transformed with pMWC or pMWCB. Each of the resulting transformants AJ13355/pMWC, AJ13355/pMWCB, AJ13399/pMWC and AJ13399/pMWCB and the parent strains were inoculated into 500 ml-volume flask containing 20 ml of culture medium comprising 40 g/L glucose, 20 g/L ammonium sulfate, 0.5 g/L magnesium sulfate heptahydrate, 2 g/L

potassium dihydrogenphosphate, 0.5 g/L sodium chloride, 0.25 g/L calcium chloride heptahydrate, 0.02 g/L ferrous sulfate heptahydrate, 0.02 g/L manganese sulfate tetrahydrate, 0.72 mg/L zinc sulfate dihydrate, 0.64 mg/L copper sulfate pentahydrate, 0.72 mg/L cobalt chloride hexahydrate, 0.4 mg/L boric acid, 1.2 mg/L sodium molybdate dihydrate, 2 g/L yeast extract, and 30 g/L calcium carbonate, and cultured at 37°C for 15 hours with shaking. After the cultivation was completed, L-glutamic acid accumulated in the culture medium and residual glucose were measured. The results are shown in Table 1.

Table 1: Accumulated amount of L-glutamic acid

Bacterial strain	Accumulated amount of L-glutamic acid (g/L)	Residual amount of Glucose (g/L)
AJ13355	0	0
AJ13355/pMWC	0.01	6.0
AJ13355/pMWCB	0.78	28.5
AJ13399	0	0
AJ13399/pMWC	2.85	0
AJ13399/pMWCB	4.71	0

L-glutamic acid productivity was observed in both *Enterobacter agromellans* AJ13355 and *Klebsiella planticola* AJ13399 by introducing a *gltA* gene. The accumulation of L-glutamic acid is more marked when a

gltA gene derived from *Brevibacterium lactofermentum* is introduced than when a *gltA* gene derived from *Escherichia coli* is introduced. A great amount of glucose remains in the case of the AJ13355/pMWCB without being consumed under the above conditions. When culture is carried out until all glucose is consumed, it is assumed that about 1.5 to 2 g/l of L-glutamic acid can be accumulated.

There was not seen a marked difference in the copy number of plasmid between AJ13355/pMWC and AJ13355/pMWCB, and between AJ13399/pMWC and AJ13399/pMWCB.

SEQUENCE LISTING

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<120> L-GLUTAMIC ACID PRODUCING BACTERIUM AND PROCESS FOR PRODUCING L-GLUTAMIC ACID

<130> P-5935

<141> 1998-10-19

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<223> Description of Artificial Sequence: primer

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aagcttatcg acgctcccct ccccaccgtt 30

[Brief Explanation of the Drawings]

[Fig. 1] It shows the construction of a plasimd pMWCB having a *gltA* gene.

[Fig. 2] It shows the construction of a plasimd having a *gltA* gene.

[Name of the Document] Abstract

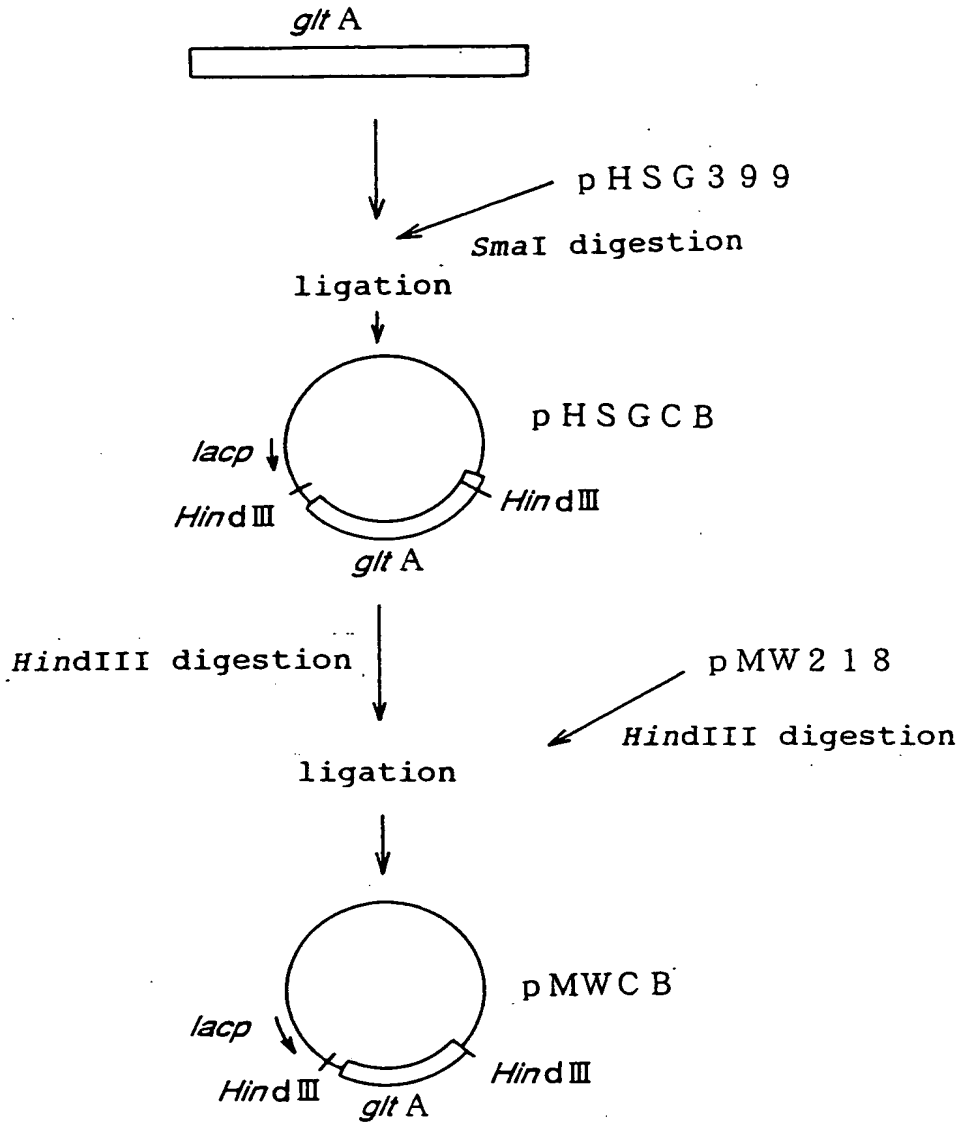
[Abstract]

[Object] It is to find a new L-glutamic acid producing bacterium having L-glutamic acid productivity in order to develop an inexpensive and efficient process for producing L-glutamic acid.

[Solving Means] L-glutamic acid is produced by culturing in a medium a microorganism belonging to enterobacteria and having L-glutamic acid productivity, into which a citrate synthase gene derived from a coryneform bacterium is introduced to produce and accumulate L-glutamic acid in the medium and collecting the L-glutamic acid from the medium.

[Drawing selected] Fig. 1

Fig.1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ligation
pHSGCB
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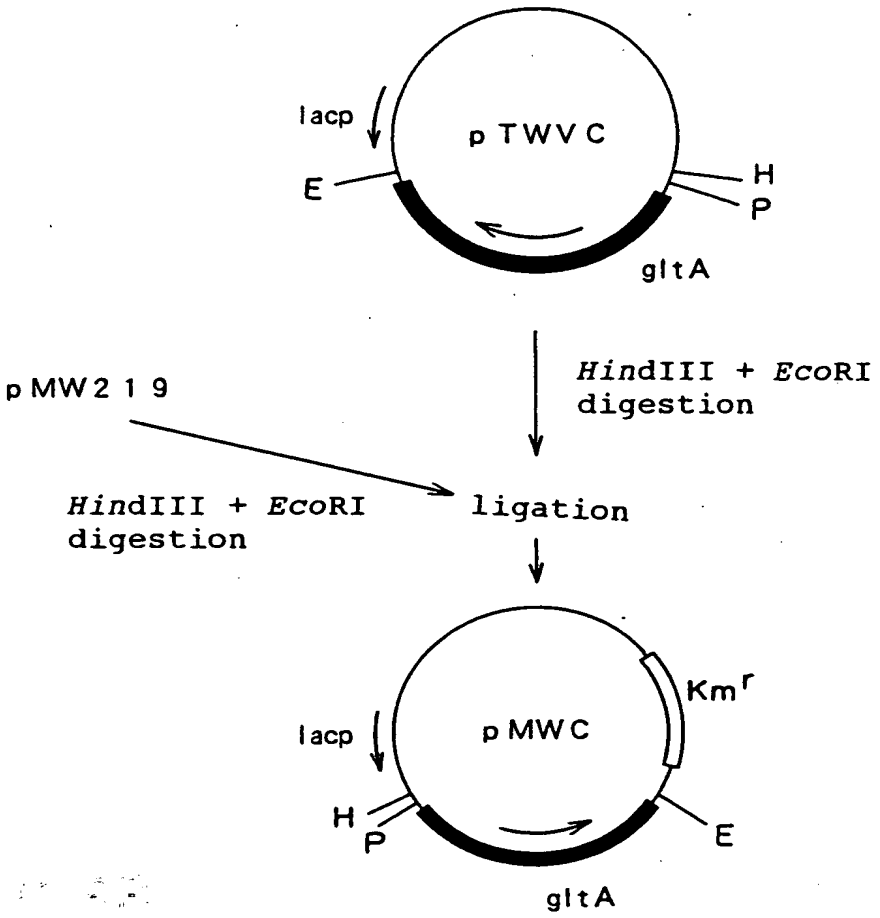
Fig.2



Hi ; HindIII
P ; PstI
E ; EcoRI